Gene 563 (2015) 24–28

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Single nucleotide polymorphism creating a variable upstream open reading frame regulates glucocorticoid receptor expression

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ARTICLE INFO

Article history:
Received 20 November 2014
Received in revised form 20 January 2015
Accepted 1 March 2015
Available online 12 March 2015

Keywords:
Glucocorticoid receptor
NR3C1
Mineralocorticoid receptor
NR3C2
Cellular stress
SNP
Upstream open reading frame

ABSTRACT

The glucocorticoid and mineralocorticoid receptors are known to play a crucial role in cellular responses to acute and chronic stress conditions. However, the influence of genetic variants and regulatory mechanisms within the glucocorticoid and mineralocorticoid receptor genes *NR3C1* and *NR3C2* is still incompletely understood. We therefore investigated putative upstream open reading frames, a motif regulating gene expression, from the 5′ untranslated regions of the predominant human glucocorticoid receptor gene *NR3C1* isoform alpha variant 1 and from the human mineralocorticoid receptor *NR3C2* variants 1 and 2. The *in silico* analysis displayed one SNP (rs10482612), being present heterozygously in about 1.2% of the world population and 1.8% of the European population (according to the NCBI database), whose minor allele 'A' creates an upstream start codon. Our functional analysis performed by reporter gene assay and quantitative real-time PCR confirmed that the minor allele 'A' of the SNP rs10482612 can indeed alter protein activity of the subsequent gene during baseline conditions and cellular stress by creating a functional uORF in the 5′UTR of the *NR3C1* transcript variant 1.

1. Introduction

The mineralocorticoid (MR) and glucocorticoid (GR) receptors are members of the steroid receptor superfamily and act as ligand-regulated transcription factors. Both the MR and the GR play pivotal roles in a broad spectrum of biologic processes such as metabolism, development, immune and stress response (Gekle et al., 2014; Heitzer et al., 2007; Wood et al., 2013; Nicolaides et al., 2010).

Clinical studies have revealed that numerous inter-individual variations can be present with respect to the MR and GR pathways. These variations can be partly explained by DNA sequence variants and regulatory motifs within the *NR3C1* (encoding the GR) and *NR3C2* (encoding the MR) genes (Breslin and Vedeckis, 1996, 1998; Geng et al., 2008; Pedersen et al., 2004; Fischer et al., 2010). In particular single nucleotide polymorphisms (SNPs) have been linked to altered physiological and pathological conditions, such as the *NR3C1* polymorphism N363S, which is associated with a higher BMI, elevated cholesterol levels and an increased risk for coronary artery disease (Lin et al., 2003; van Rossum and Lamberts, 2004). Likewise, quite a few SNPs that influence stress response within the organism through altered corticosteroid sensitivity could be identified in both the *NR3C1* and *NR3C2* genes. The molecular mechanism behind these associations remains mainly undiscovered so far. Exceptions are, for instance, the SNP A3669G and the ATTA motif of the *NR3C1* gene that have been shown to exert their effects through modifying RNA stability (Derijk et al., 2001; Syed et al., 2006). Yet, most studies focus on genetic variants in the exonic and intronic gene regions. No study could be found in the literature that searched for regulatory motifs and therewith associated SNPs in the untranslated region (UTR) influencing human *NR3C1* and *NR3C2* gene expression. However, it is well known by now that the UTR might harbour regulatory gene expression motifs which are capable of governing the subsequent gene. One of these UTR motifs is upstream open reading frames (uORFs) which affect protein translation by *e.g.* blocking the translational machinery or triggering nonsense mediated decay (Barbosa et al., 2013; Calvo et al., 2009). The involvement of uORFs in genetic diseases such as hereditary thrombocythaemia underlines the importance of this gene motif. Furthermore, several SNPs

Abbreviations: A, adenosine; C, cytidine; G, guanosine; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; mRNA, messenger RNA; *NR3C1*, glucocorticoid receptor gene; *NR3C2*, mineralocorticoid receptor gene; qPCR, quantitative real-time polymerase chain reaction; SNP, single nucleotide polymorphism; T, thymidine; uORF, upstream open reading frame; UTR, untranslated region

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http://dx.doi.org/10.1016/j.gene.2015.03.015
0378-1119/

creating variable uORFs have already been identified in different genes (Barbosa et al., 2013; Calvo et al., 2009). In the murine glucocorticoid receptor transcript variant 1A a functional uORF peptide was reported by Diba et al. (2001).

2. Material and methods

2.1. In silico analysis of the 5′UTR of NR3C1 and NR3C2 variants

According to the NCBI database, five respective two different transcript variants exist for *NR3C1* and *NR3C2* (http://www.ncbi.nlm.nih.gov/gene). As for *NR3C1* we focused on the 5′UTR transcript variant 1 (NM_000176.2) of the isoform alpha, encoding the predominant physiological isoform. Regarding *NR3C2*, the 5′UTR as well as the first part of the coding region of the two variants are identical in sequence. The 5′UTR of *NR3C1* transcript variant 1 (NM_000176.2) and *NR3C2* variants 1 (NM_000901.4) and 2 (NM_001166104.1), was searched for putative uORFs with the StarORF Finder (http://star.mit.edu/orf/runapp.html).

2.2. Plasmid construction

The two luciferase vectors pGL4.10 and pGL4.74 were purchased from Promega (Mannheim, Germany). The TK-Promoter sequence was cut out of pGL4.74 with *Kpn*I and *Xho*I (Fermentas, St. Leon-Rot, Germany) after generating a *Xho*I restriction site at position bp 783 using GeneArt site-directed mutagenesis system (Invitrogen, Karlsruhe, Germany). After *Kpn*I and *Xho*I digestion of pGL4.10, the TK-Promoter sequence was ligated into the multiple cloning site of pGL4.10 upstream of the firefly luciferase coding sequence. The 5′UTR inserts of the *NR3C1* variant 1 and *NR3C2* genes were synthesized (MWG Eurofins, Ebersberg, Germany) and cloned into pGL4.10 with *Xho*I and *Nco*I directly between the TK-Promoter and the firefly luciferase coding sequence. After cloning the inserts were confirmed by sequencing. To create constructs lacking a certain uORF, the ATG of the uORF was mutated to TTG. For *NR3C1* variant 1 seven constructs were created: six constructs that differ in terms of their mutated uORF start codons, and one construct harbouring an in-frame stop codon (tga) three bases downstream of the uORF start codon (*NR3C*1-1.7) for the additional luciferase experiment.

For *NR3C2* two constructs were created that differ in terms of their mutated uORF start codons. Sequence details of the created constructs are displayed in Table S1.

2.3. Cell culture

The human embryonic kidney (HEK) cells 293 were purchased from Cell lines service (Eppelheim, Germany). Culturing of the cells was performed in T25 flasks in monolayer with DMEM (Sigma Aldrich, Hamburg, Germany) containing 4.5 g/l glucose, 1% L-Glutamine, 10% foetal bovine serum (FBS) and 1% Penicillin/Streptomycin (Invitrogen, Karlsruhe, Germany). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. For all experiments HEK293 cells were seeded with 2×10^5 cells in 24 well plates (Greiner Bio-One, Frickenhausen, Germany). Co-transfection was performed with the reporter plasmid pGl4.10 + TK containing the 5′UTR constructs of *NR3C1* variant 1 or *NR3C2*, respectively, and the control plasmid pGL4.74 at a ratio of 20:1 using TransIT®-LT1 Transfection Reagent (MoBiTec, Göttingen, Germany) with 24 h duration of transfection after seeding.

2.4. Baseline and stress condition tests

Baseline test: 24 h after co-transfection culture medium was replaced and the HEK293 cells were kept for 24 h in the incubator at 37 °C and 5% CO_2. Hypoxia stress test: 24 h after co-transfection culture medium was replaced and the HEK293 cells were kept in a hypoxia chamber containing 95% nitrogen/5% CO_2 for 24 h according to the manufacturer's protocol (Anaerocult C mini, Merck, Darmstadt, Germany) prior to luciferase assay. No glucose stress test: 24 h after co-transfection culture medium was replaced by DMEM containing no glucose, but FBS, 1% L-Glutamine and 1% Penicillin/Streptomycin (Invitrogen, Karlsruhe, Germany) for 24 h prior to luciferase assay or qPCR. Hyperthermia test: 24 h after co-transfection culture medium was replaced and the HEK293 cells were kept for 24 h at 41 °C in the incubator prior to luciferase assay. Test conditions were adapted from Fischer et al. (2008) and Lohse et al. (2011).

2.5. Luciferase assay

The firefly and renilla luciferase activities were measured with the TRiStar LB941 (Berthold Technologies, Bad Wildbad, Germany) by the Dual-Glow® luciferase assay system (Promega, Mannheim, Germany) according to the manufacturer's protocol. The ratio of firefly luciferase and renilla luciferase activity was determined and normalized to pGL4.10 + TK. The results for the various 5′UTR constructs are given as fold protein activity change to pGL4.10 + TK.

2.6. Quantitative real-time PCR

qPCR was performed under hypoxic condition for *NR3C1* variant 1 constructs *NR3C1*-1.1, *NR3C1*-1.5 and *NR3C1*-1.6. Co-transfection with the various constructs was performed as described above. RNA was extracted by using a Qiagen RNeasy plus kit, including DNase treatment of 15 min, according to the manufacturer's protocol (Qiagen, Hilden, Germany). First-strand cDNA synthesis was carried out using 1.6 μg of RNA from each transfection as starting material (QuantiTect reverse transcription kit, Qiagen, Hilden, Germany). qPCR was performed targeting firefly luciferase (target) and renilla luciferase (control) coding sequence using primers firefly-fwd 5′- TGCAACACCCCAACATCTTC-3′ and firefly-rev 5′- CCTTTAGGCACCTCGTCCAC-3′; renilla-fwd 5′- AATGGCTCATAT CGCCTCCT-3′ and renilla-rev 5′- CACGACACTCTCAGCATGGA-3′. The reactions were carried out in 20 μl volumes containing 10 μl SsoFast™ EvaGreen® Supermix (Bio-Rad, Munich, Germany), 125 nM of each primer, 2 μl molecular biology grade water and 6 μl of each template after cDNA synthesis. Thermal cycling consisted of denaturation (98 °C for 5 s), annealing and extension (60 °C for renilla; 62 °C for firefly for 5 s), performed in 50 cycle steps. Melting curve analysis ranged from 65 °C to 95 °C with 0.5 °C intervals.

2.7. Statistical analysis

All luciferase and qPCR experiments were repeated independently three times with triplicate technical samples. The Livak calculation method corrected for amplification efficiencies was applied for qPCR data analysis (Livak and Schmittgen, 2001). A two-tailed t-test was used to compare the values of the target samples and the control samples. A p value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Results of the in silico analysis

Three putative uORFs, the first two overlapping, were detected in the 5′UTR of *NR3C1* isoform alpha variant 1 (NM_000176.2). Within this 5′ UTR a validated SNP (rs10482612) is present (http://www.ncbi.nlm.nih.gov/snp/?term=rs10482612), with the minor allele, A′ creating the start codon for the first uORF. The stop codon of the first uORF is located within the second uORF.

In silico analysis for the 5′UTR of *NR3C2* (NM_000901.4) identified one putative uORF with 132 nt length, overlapping the main coding sequence by 32 nt (Fig. 1).